Exhibit 4

Generation Ex Vivo of TGF-β-Producing Regulatory T Cells from $CD4^+CD25^-$ Precursors[1]

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Previously we reported that TGF-β has an important role in the generation and expansion of human "professional" $CD4^+CD25^+$ regulatory T cells in the periphery that have a cytokine-independent mechanism of action. In this study we used low-dose staphylococcal enterotoxin to induce T cell-dependent Ab production. We report that TGF-β induces activated $CD4^+CD25^-$ T cells to become Th3 suppressor cells. While stimulating $CD4^+$ cells with TGF-β modestly increased expression of CD25 and intracellular CTLA-4 in primary cultures, upon secondary stimulation without TGF-β the total number and those expressing these markers dramatically increased. This expansion was due to both increased proliferation and protection of these cells from activation-induced apoptosis. Moreover, adding as few as 1% of these TGF-β-primed $CD4^+$ T cells to fresh $CD4^+$ cells and B cells markedly suppressed IgG production. The inhibitory effect was mediated by TGF-β and was also partially contact dependent. Increased TGF-β production was associated with a decreased production of IFN-γ and IL-10. Depletion studies revealed that the precursors of these TGF-β-producing $CD4^+$ suppressor cells were CD25 negative. These studies provide evidence that $CD4^+CD25^-$ regulatory cells in human blood consist of at least two subsets that have TGF-β-dependent and independent mechanisms of action. TGF-β has an essential role in the generation of both of these T suppressor cell subsets from peripheral T cells. The ability to induce $CD4^+$ and $CD8^+$ cells to become regulatory cells ex vivo has the potential to be useful in the treatment of autoimmune diseases and to prevent transplant rejection. ***The Journal of Immunology,*** **2002, 169: 4183–4189.**

Transforming growth factor-β is a multifunctional cytokine with both positive and negative effects on the immune system (1). While its inhibitory effects are well known (2), this cytokine can also induce IL-2-activated human CD4 and $CD8^+$ T cells to develop potent down-regulatory effects. Previously, we have reported that TGF-β can induce mitogen-stimulated $CD8^+$ cells to suppress T cell-dependent Ab production (3, 4). We have also observed that TGF-β can generate $CD4^+CD25^+$ cells that have very potent contact-dependent suppressive effects on alloactivated or $CD8^+$ T cells (5). These suppressive effects were similar to, if not identical with, the "professional" $CD4^+CD25^+$ T cells described by others (6, 7). Here we report that TGF-β costimulates $CD4^+CD25^-$ cells and induces them to become potent suppressors of Ab production by a TGF-β-dependent mechanism of action.

To study T cell/B cell interactions, we have used model systems where the confounding effects of additional APCs can be excluded. In investigating how $CD8^+$ T cells become suppressor cells, we used a mitogenic combination of anti-CD2 Abs for this purpose (4). More recently, Stohl and Elliott (8) have found that the bacterial superantigen staphylococcal enterotoxin B (SEB)[3] has a similar effect. This superantigen binds to T cells expressing Vβ8 and HLA-DR on B cells and in high doses eliminates the latter cells by activation-induced apoptosis. However, in low doses SEB induces Ab production by a direct interaction of T cells and B cells without additional accessory cells (8). We have found that the magnitude of this response is controlled by TGF-β produced by cells in the immediate microenvironment. Exposure of $CD4^+$ cells to TGF-β at the time they were activated with SEB altered the genetic program of these cells. Upon restimulation, these $CD4^+$ T cells expanded, expressed high levels of CD25 and CTLA-4, and developed potent TGF-β-dependent suppressive activity.

Materials and Methods

The following Abs were used: anti-CD3, anti-CD8, anti-CD20, anti-CD25, anti-CTLA-4, anti-CD122, control IgG (all from BD PharMingen, San Diego, CA); anti-CD8 (OKT8), anti-CD11b (OKM1), anti-CD74 (L243) (all hybridomas from American Type Culture Collection, Manassas, VA); anti-CD16 (3G8, kindly provided by Dr. J. Unkeless, Mount Sinai Medical School, New York, NY); anti-CD103 (DAKO, Carpinteria, CA); and anti-TGF-β (R&D Systems, Minneapolis, MN). SEB was purchased from Sigma-Aldrich (St. Louis, MO). TGF-β1 and IL-2 were from R&D Systems.

Lymphocyte isolation

PBMC were prepared from heparinized venous blood of healthy adult volunteers by Ficoll-Hypaque (Atlanta Biologicals, Norcross, GA) density gradient centrifugation. To prepare PBL, PBMC were added to a continuous Percoll (Pharmacia, Piscataway, NJ) density gradient and the high-density fraction was collected (9). T cells were prepared by immediate rosetting with 2-aminoethylisothiouronium bromide-treated SRBC (10). T cells were further purified from rosetting cells by staining with Abs to CD16, CD74, and CD11b and depleting reactive cells using immunomagnetic beads (Dynal Biotech, Great Neck, NY). The percentage of $CD3^+$ cells in this fraction was usually >96%.

$CD4^+$ cells were prepared from T cells that were stained with Abs to CD8 by negative selection using immunomagnetic beads. Purity of $CD4^+$ cells was usually 95%. CD25 depleted CD4 T cells were prepared from $CD4^+$ T cells by cell sorting. Before sorting, the $CD4^+CD25^-$ population was ~3.5% among total $CD4^+$ T cells. After sorting, the $CD4^+CD25^+$ population was <0.3%. In some experiments $CD8^+$ cells were prepared by negative selection (4). To obtain B cells, nonrosetting PBMC were treated

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[3] Abbreviations used in this paper: SEB, staphylococcal enterotoxin B; SPC, superantigen-presenting cell; LME, L-leucine methyl ester.

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with 5 mM L-leucine methyl ester (LME) for depletion of monocytes and NK cells (11). These cells were stained with Abs to CD3, CD16, and CD11b and depleted of reactive cells by immunomagnetic beads. The resulting population was >90% $CD20^+$ and <0.5% $CD3^+$.

Generation and assay of regulatory $CD4^+$ cells

$CD4^+$ cells (2×10^6) and irradiated B cells as superantigen-presenting cells (SPC) (2×10^6) were cultured with SEB (0.01 ng/ml) in the presence or absence of TGF-β1 (0.1–10 ng/ml) in AIM V serum-free medium (Invitrogen, Carlsbad, CA) for 5–6 days in 24-well plates (Multiwell; BD Labware, Franklin Lakes, NJ). Serum-free medium was used because TGF-β binds to various serum components (12). The cells were washed and various numbers were added to fresh autologous $CD4^+$ cells (5×10^4/well) and B cells (5×10^4/well) in 96-well flat-bottom microtiter plates (Falcon, Lincoln Park, NJ) and cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% heated-inactivated FCS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin (Invitrogen), 100 g/ml streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 mM Na-pyruvate (Invitrogen), and 10 mM HEPES (Invitrogen). After culture in complete medium for 7–10 days, the supernatants were harvested and IgG content was determined by ELISA (3). The variation between triplicate wells was usually <10%. In some experiments, proliferation in secondary cultures was assessed by uptake of tritiated thymidine added for the final 18 h and assessment of cell death by annexin V staining as performed by flow cytometry according to instructions from the manufacturer (BD PharMingen).

Measurement of cytokine production

Primed $CD4^+$ cells were extensively washed and restimulated with 0.01 ng/ml SEB for 24, 48, and 72 h in serum-free AIM V serum for TGF-β production, and with complete medium for production of other cytokines. In some experiments, IL-2 (10 U/ml) was added to the cultures. Active TGF-β was determined by mink lung epithelial cells transfected with a luciferase gene construct (13). Several concentrations of TGF-β were included to generate a standard curve, and the variation between triplicate samples was always <10%. Supernatants were also tested in duplicate using ELISA kits for IL-4, IL-10, and IFN-γ (BioSource International, Camarillo, CA). The limits of detection of the assays performed were 7.8–500 pg/ml (for IL-4 and IL-10) and 7.8–1000 pg/ml (for IFN-γ).

Transwell studies

$CD4^+$ T cells and CD25-depleted $CD4^+$ T cells were primed with SEB with or without TGF-β as described above. After 5–6 days, these cells were extensively washed, mixed with fresh $CD4^+$ cells in different ratios (1:5, 1:20, and 1:100), and added to the wells of a 24-well plate containing $CD4^+$ cells, B cells, and SEB. In some wells, the conditioned $CD4^+$ cells and irradiated SPC were separated from responder cells by the insert of a Transwell plate (Corning Costar, Cambridge, MA). Supernatants were collected after 10 days and assayed for IgG content by an ELISA.

Immunofluorescence analysis

Cell surface Ag expression on effector $CD4^+$ T cells was determined by flow cytometry. $CD4^+$ T cells (10^5) were labeled with FITC-conjugated (anti-CD4) and PE-conjugated (anti-CD25) mAbs. After 20 min at 4°C in PBS with 0.1% BSA and 0.02 mM NaN_3, the cells were washed and analyzed on a FACStar^{Plus} flow cytometer using CellQuest software (BD Biosciences, San Jose, CA).

For staining of intracellular CTLA-4, activated $CD4^+$ T cells were harvested and washed twice with PBS. After staining for surface markers, they were fixed and permeabilized with 4% paraformaldehyde and 0.1% saponin buffer for 20 min. After two washes, the cells were incubated with normal mouse serum to inhibit nonspecific binding followed by incubation for 20 min with PE-anti-CTLA-4 or PE-conjugated control mAbs (cIgG). All staining was performed on ice and at least 10,000 viable cells were analyzed.

Statistical analysis

Significance of the results was analyzed by Student's *t* test performed with GraphPad Prism software (GraphPad, San Diego, CA).

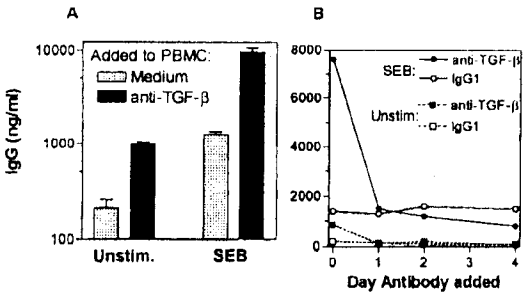

FIGURE 2. Effect of anti-TGF-β on SEB-induced IgG synthesis. *A*, PBMC (1×10^5/well) were stimulated with SEB (0.01 ng/ml) with or without anti-TGF-β (10 μg/ml) for 10 days as in Fig. 1. *B*, Anti-TGF-β or control IgG1 was added at the days indicated and the effect on IgG production is shown. These studies have been performed at least four times with similar results.

Results

TGF-β controls SEB-induced T cell-dependent Ab production

Our initial studies suggested that the magnitude of IgG induced by low-dose SEB is controlled by TGF-β produced by cells in the immediate microenvironment. In cultures containing PBMC or PBL, the dose of SEB that induced IgG production correlated inversely with the amount of TGF-β detected (Fig. 1, *A* and *B*). By contrast, throughout this concentration range of SEB, IgG production remained high and levels of TGF-β were minimal in cultures containing purified $CD4^+$ cells and B cells. Because NK cells and monocytes are the major sources of TGF-β in PBMC (4), we lysed these cells with LME and observed a marked increase in IgG production. However, adding back nanomolar concentrations of

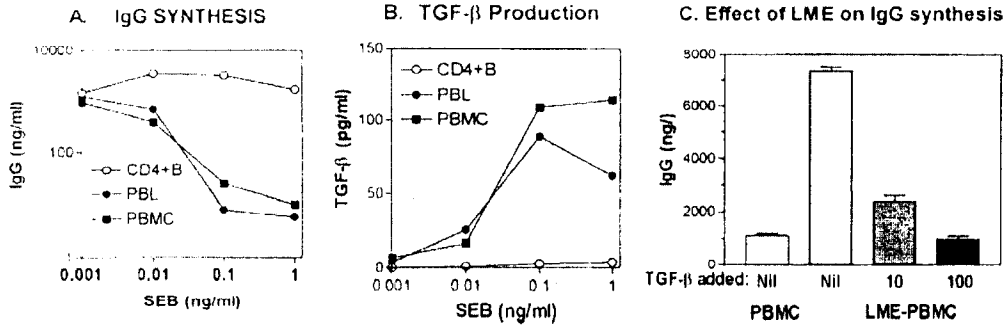

FIGURE 1. TGF-β regulates T cell-dependent Ab production induced by low-dose superantigen. *A*, PBMC or PBL (1×10^5/well), or purified CD4 and B cells (5×10^4 well), in 96-well plates were stimulated with graded doses of SEB for 10 days and the supernatants were examined for IgG content. Unstimulated cells produced 95 ng/ml IgG. *B*, Supernatants from parallel cultures were harvested at 48 h and assayed for active TGF-β. *C*, PBMC were stimulated before and after treatment with LME. A TGF-β (10 or 100 pg/ml) was added as shown and stimulated with SEB as above. Each of these studies was performed at least three times with similar results.

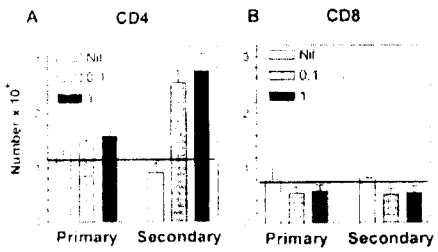

FIGURE 3. $CD4^+$ cells primed with TGF-β are selectively expanded when restimulated. T cells (2×10^6/well) and an equal number of irradiated B cells as SPC were cultured with SEB (0.01 ng/ml) with or without TGF-β (0.1 or 1 ng/ml) for 5 days. The cells were washed and restimulated with SEB (0.01 ng/ml) without TGF-β for 3 days. *A*, Cells stained by anti-CD4 Abs. *B*, Cells stained by anti-CD8 Abs. The bars indicate the mean and SEM of triplicate cultures. The horizontal lines indicate the starting number of $CD4^+$ and $CD8^+$ cells. This study is representative of nine separate experiments.

TGF-β to the LME-treated PBMC decreased IgG production to background levels (Fig. 1C).

Studies with a neutralizing anti-TGF-β Ab provided further evidence for a role for TGF-β in the regulation of SEB-induced IgG production. The addition of anti-TGF-β resulted in a modest but significant increase in background IgG by PBMC and a 10-fold increase in IgG production by SEB-stimulated cells (Fig. 2*A*). This enhancement of IgG production was lost if anti-TGF-β was added 1 day after the cells were stimulated (Fig. 2*B*). This result suggested an early effect on T cell activation rather than later B cell differentiation.

Priming of T cells with TGF-β enables $CD4^+$ cells to expand more rapidly after restimulation and protects them from activation-induced cell death

The next series of experiments revealed several costimulatory effects of TGF-β on $CD4^+$ cells. Others have reported that after mitogen activation of T cells in the presence of TGF-β there is marked enhancement of proliferation upon restimulation (14). We have confirmed this finding with SEB and have found that, in a mixed population of human peripheral blood $CD4^+$ and $CD8^+$ T lymphocytes, TGF-β had a selective effect on $CD4^+$ cells (Fig. 3*A*). After restimulation of TGF-β-primed cells with low-dose SEB, the absolute number of $CD4^+$ but not $CD8^+$ cells markedly increased (Fig. 3*B*). This result differs from the findings of others who reported positive effects of TGF-β on mouse $CD8^+$ cells (15). TGF-β also significantly increased the number of purified $CD4^+$ cells in primary cultures ($p = 0.04$), but this increase was modest.

In the experiment shown in Fig. 4, the increased expansion of TGF-β-primed $CD4^+$ cells after restimulation can be attributed to both enhanced proliferation and protection from activation-induced cell death. In this experiment $CD4^+$ cells were primed with or without TGF-β for 6 days, rested for 1 day, and then restimulated with low-dose SEB without TGF-β. Three days later uptake of tritiated thymidine by these cells was 3-fold greater than control $CD4^+$ cells. Within 6 days after restimulation almost one half of control $CD4^+$ cells were undergoing apoptosis. By contrast, <10% of TGF-β-primed $CD4^+$ cells were annexin V positive.

Activation of $CD4^+$ cells in the presence of TGF-β also increased expression of CD25 and CTLA-4. This increase was generally modest in primary cultures but was marked after restimulation without TGF-β. In a representative experiment shown in Fig. 5, stimulation of $CD4^+$ cells with low-dose SEB with TGF-β for 6 days increased the cluster of $CD25^+CTLA\text{-}4^+$ double stained cells from 13 to 24%. Three days later after restimulation without TGF-β, 70% of TGF-β-primed T cells expressed both of these markers in contrast to 53% of controls. In addition, the numbers of TGF-β-primed $CD4^+$ cells had more than doubled in contrast to control $CD4^+$ cells, which had increased by only 50%. $CD4^+CD25^+$ T cells that strongly express $CTLA\text{-}4^+$ bear the phenotype of regulatory T cells (7).

Cytokines produced by $CD4^+$ cells primed with TGF-β

$CD4^+$ T cells primed with TGF-β had a much greater capacity to produce the active form of this cytokine. While TGF-β produced by T cells is generally in the latent precursor form (16, 17), $CD4^+$ cells primed in the presence of TGF-β and restimulated with SEB produced greater amounts of active TGF-β in comparison with controls (Fig. 6). This effect was markedly accentuated by including IL-2 in secondary cultures where a dose-dependent effect of TGF-β was documented. Priming $CD4^+$ cells with 10 ng/ml

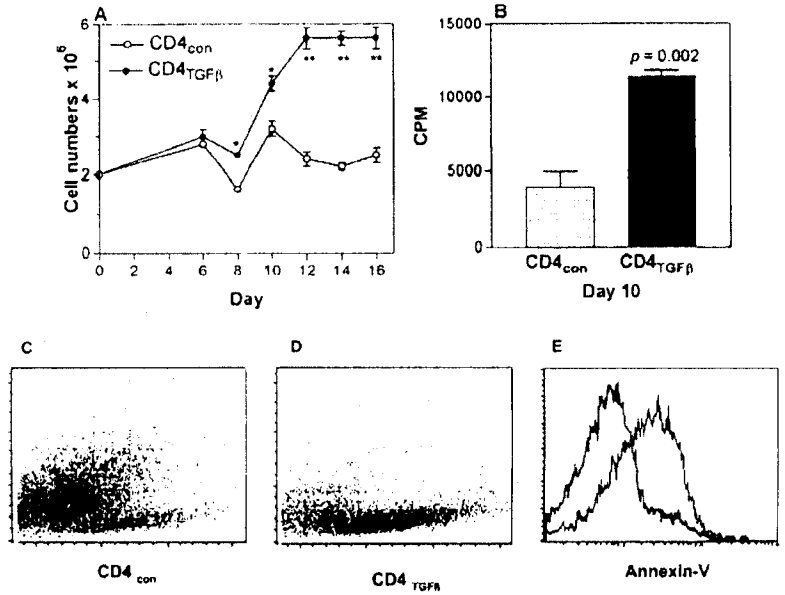

FIGURE 4. Expansion of TGF-β-primed $CD4^+$ cells after restimulation and protection from activation-induced apoptosis. Two million $CD4^+$ cells and an equal number of irradiated B cells were cultured for 6 days in serum-free medium with SEB (0.01 ng/ml) and TGF-β1 (10 ng/ml) ($CD4_{TGF\text{-}\beta}$) or without this cytokine ($CD4_{con}$). The cells were washed, resuspended in medium containing 10% FCS, and rested overnight. Two million $CD4^+$ cells and SPC were then restimulated with low-dose SEB plus IL-2 (10 U/ml). *A*, Sequential cell counts of triplicate cultures. The mean ± SEM are indicated. Significant differences between $CD4_{con}$ and $CD4_{TGF\text{-}\beta}$ (*t* test) are shown (*, $p < 0.05$; **, $p < 0.01$). *B*, Tritiated thymidine uptake was assessed 3 days after restimulation. Six days after restimulation, viability of $CD4_{con}$ and $CD4_{TGF\text{-}\beta}$ was assessed by flow cytometry. *C* and *D*, Cell size (forward scatter) on the abscissa and cell granularity (side scatter) on the ordinate. *E*, Annexin V staining. Gray line, $CD4_{TGF\text{-}\beta}$; black line, $CD4_{con}$. These data are representative of four experiments.

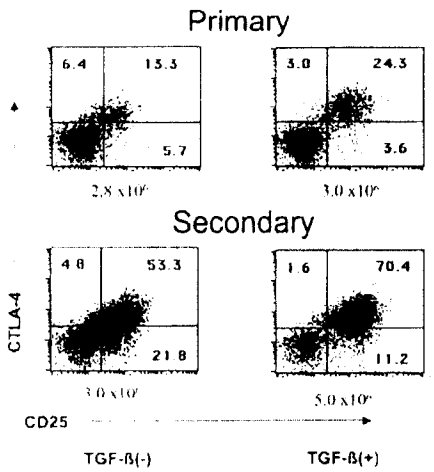

FIGURE 5. Costimulatory effects of TGF-β on CD25 and CTLA-4 expression by CD4⁺ cells. CD4⁺ cells (2×10^6) with SPC were stimulated with low-dose SEB with or without TGF-β (10 ng/ml) for 6 days. CD4⁺ cells (2×10^6) were then restimulated with SEB as described above for an additional 3 days. *Upper panels*, CD4⁺ cells double stained for surface CD25 and intracellular CTLA-4 after primary culture. *Lower panels*, The effects of restimulation. The cell numbers and percentages that stained for each marker are indicated. The results are representative of six experiments.

TGF-β resulted in a much greater amount of active TGF-β than cells primed with 0.1 ng/ml TGF-β (Fig. 6).

Production of IFN-γ and IL-10 by restimulated TGF-β-primed CD4⁺ cells was significantly lower than that of control CD4⁺ cells. The decreased IFN-γ production was a direct effect of TGF-β because it was abolished by anti-TGF-β (Fig. 7*A*). Although the decrease in IL-10 production was related to the dose of TGF-β used in primary cultures, it was not reversed by anti-TGF-β in secondary cultures (Fig. 7*B*). IL-4 production was also assessed and differences in the production of this cytokine were not observed in three separate experiments (data not shown). Because only TGF-β and not IL-4 or IL-10 was produced by these cells in large amounts, we concluded that we had generated the Th3 cells previously described by Weiner et al. (18).

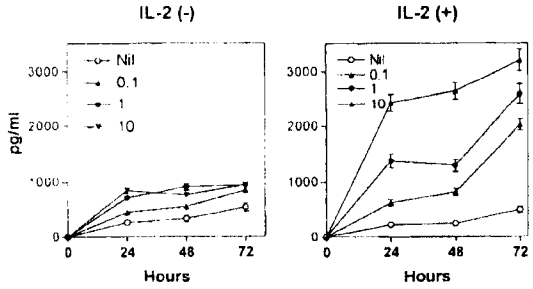

FIGURE 6. CD4⁺ cells primed with TGF-β produce the active form of this cytokine upon restimulation. CD4⁺ cells (2×10^6/well) were stimulated with low-dose SEB for 6 days with the concentrations of TGF-β shown in nanograms per milliliter. The cells were then restimulated for 1–3 days with SEB in the presence or absence of IL-2 (10 U/ml). The supernatants were then tested for active TGF-β. The results shown indicate the mean ± SEM of three independent experiments. In cultures without IL-2, the mean values of TGF-β produced by priming CD4⁺ cells with 1 or 10 ng/ml were significantly greater than control CD4⁺ cells ($p = 0.05$–0.006) at all times studied. When CD4⁺ cells were restimulated with IL-2, TGF-β production by CD4⁺ T cells primed with this cytokine were even greater, and all values were significantly higher than control CD4⁺ cells ($p < 0.002$). TGF-β carry-over from primary cultures was excluded by examining the supernatants of primed CD4⁺ cells that were not restimulated with SEB.

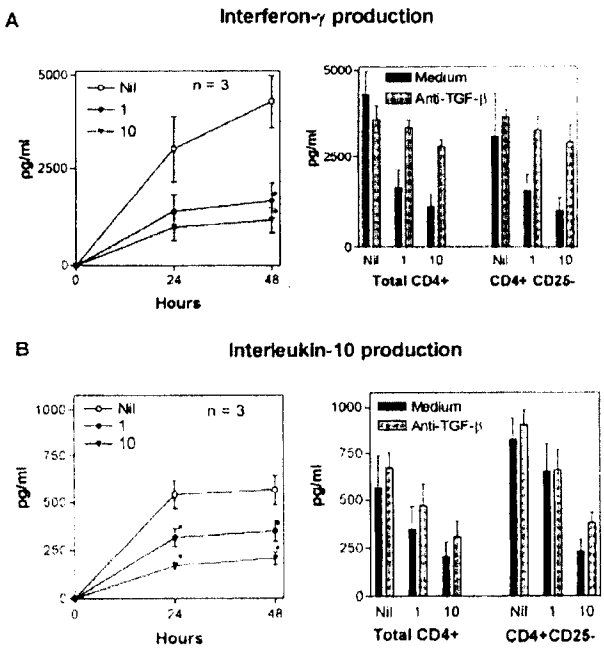

FIGURE 7. IFN-γ and IL-10 production by CD4⁺ cells conditioned with TGF-β. CD4⁺ cells (2×10^6/well) with SPC were stimulated with low-dose SEB and the doses of TGF-β indicated for 6 days and restimulated with SEB. *A*, Time course of IFN-γ production and effect of anti-TGF-β. *B*, Similar studies of IL-10 production. The mean ± SEM of three separate experiments is shown. Mean values of cytokines produced by TGF-β-primed CD4⁺ cells were significantly different from control CD4⁺ cells (*, $p < 0.05$).

The suppressive effects of CD4⁺ cells primed with TGF-β and precursor phenotype of these cells

Consistent with its known immunosuppressive effects (1), treatment of CD4⁺ cells with TGF-β for 48 h abolished the capacity of these cells to provide B cell help for Ab production (data not shown). However, even more interesting is the fact that the addition of TGF-β-primed CD4⁺ cells to fresh CD4 and B cells markedly suppressed SEB-induced IgG production. In nine separate experiments, evaluating various ratios of CD4 regulatory cells to helper cells, the addition of 5% TGF-β-treated CD4⁺ cells to fresh CD4⁺ cells had marked suppressive effects. In five of these experiments, the addition of only 1% of the TGF-β-primed CD4⁺ cells suppressed IgG production by >50%. The mean value of the suppressive activity is shown in Fig. 8*A*. This inhibition was completely abolished by anti-TGF-β Abs (Fig. 8*B*). With CD4 control cells anti-TGF-β had no effect on the production of IgG (Fig. 8*C*).

We have reported previously that TGF-β can induce activated naive CD4⁺ cells to become potent, contact-dependent CD4⁺CD25⁺ regulatory cells (5). The properties of these cells were similar to, if not identical with, the murine "professional" CD4⁺ cells described by others (7). The activity of these suppressor cells was not affected by anti-TGF-β or anti-IL-10. Because depletion of CD25⁺ cells from naive CD4⁺ cells abrogated the development of this suppressive activity (5), the precursors of the CD4⁺ professional regulatory cells apparently express CD25 constitutively. Therefore, we considered that the precursors of the Th3

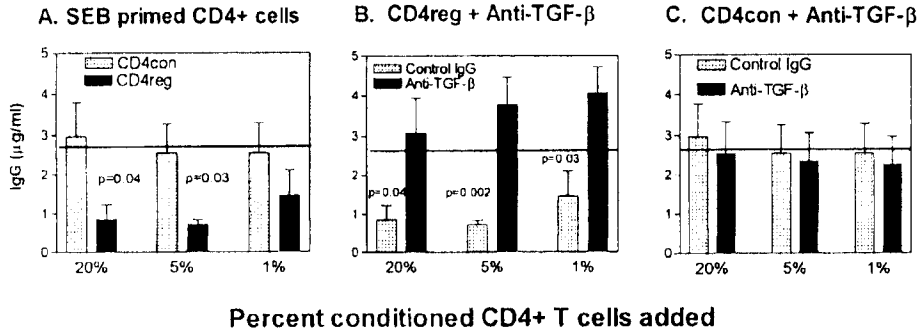

FIGURE 8. $CD4^+$ T cells primed with TGF-β develop suppressive activity mediated by this cytokine. $CD4^+$ cells and SPC (2×10^6/well) were stimulated with low-dose SEB with 1 ng/ml TGF-β (CD4reg) or without TGF-β (CD4con) for 6 days. *A*, These cells were mixed with fresh CD4 and B cells in the percentages indicated and stimulated with SEB for an additional 10 days. *B*, Some cultures contained CD4reg and anti-TGF-β (10 μg/ml) or a similar amount of an isotype-matched control IgG. *C*, Other cultures contained CD4con and anti-TGF-β. The mean IgG values ± SEM from nine experiments are shown. The horizontal line indicates the mean IgG value produced by SEB-stimulated CD4 and B cells without additional CD4 primed cells. Values of *p* are indicated.

cells described in this study were conventional resting $CD4^+$ cells and depleted $CD25^+$ cells from total $CD4^+$ cells. These $CD4^+CD25^-$ cells were stimulated with low-dose SEB in the presence of TGF-β.

The phenotype and functional properties of SEB-stimulated $CD4^+CD25^-$ cells were indistinguishable from total $CD4^+$ cells. As before, priming with TGF-β resulted in a modest increase in the total cell number and those expressing CD25 and CTLA-4 in primary cultures (Fig. 9*A*). Upon restimulation with SEB there was a marked increase in each of these populations (Fig. 9*B*), and these cells developed suppressive activity that was neutralized by anti-TGF-β (Fig. 9*C*). $CD4^+CD25^-$ cells primed with TGF-β also produced increased levels of TGF-β after restimulation with SEB (Fig. 10). The only difference noted was in the optimal dose of TGF-β required for conditioning. With total $CD4^+$ cells, maximal costimulatory and suppressive effects were noted with concentrations of TGF-β between 0.1 and 1 ng/ml. With CD25-depleted $CD4^+$ cells, maximal production of TGF-β and suppressive effects required 10 ng/ml (Figs. 9*C* and 10).

Role of cell contact in suppression of $CD4^+$ cells conditioned with TGF-β

Although suppression of IgG synthesis was mediated by TGF-β, separation of the $CD4^+$ regulatory cells from the responder cells by Transwells revealed a surprising result. With high ratios of suppressor cells to responder cells there was a partial loss of suppression with cell separation, but a complete loss was observed when the number of regulatory cells was reduced. In the experiment shown in Fig. 10, the suppressive activity by CD25-depleted $CD4^+$ cells was even greater than that of total $CD4^+$ cells. In other experiments the suppressive activity of each population was similar (Fig. 11).

We considered the possibility that TGF-β released by $CD4^+$ cells in the Transwell became bound to the membrane. However, studies in which we added various concentrations of TGF-β to either side of the Transwell and measured TGF-β outside the Transwell excluded this possibility. These studies suggested that high levels of TGF-β made by large numbers of regulatory cells could inhibit IgG production. With lower levels made by a smaller numbers of cells, the cytokine acted at a short distance and cell contact was needed for this suppressor effector activity.

Discussion

This report provides further evidence that TGF-β has an important role in the generation of regulatory T cells in addition to its well-known inhibitory activities on effector cell function. Here we have documented that TGF-β made by cells in the immediate microenvironment controls the T cell response to the bacterial superantigen

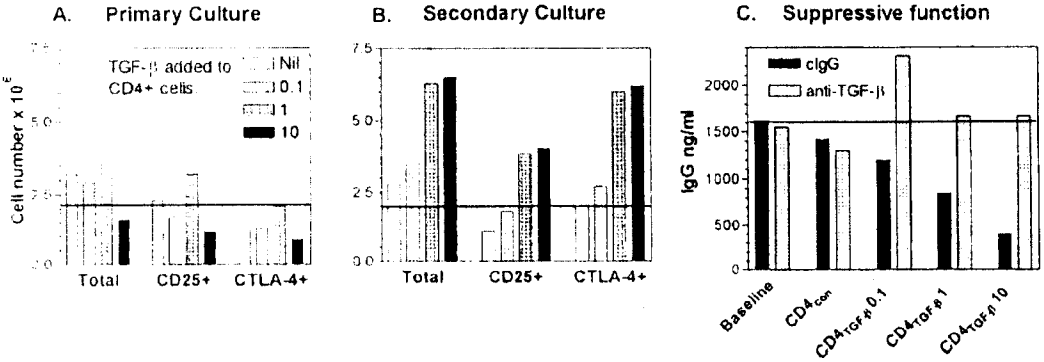

FIGURE 9. Phenotype and function of $CD4^+CD25^-$ cells conditioned with TGF-β. After depletion of $CD25^+$ cells by cell sorting, $CD4^+CD25^-$ cells and SPC (2×10^6/well) were stimulated with low-dose SEB for 6 days with or without graded concentrations of TGF-β. *A*, The total number of $CD4^+$ cells and those expressing CD25 and CTLA-4 in primary culture. *B*, The phenotype of TGF-β-primed and control $CD4^+$ cells after restimulation for 3 days. *C*, Suppressive effects of the addition of 4% of $CD4^+$ cells primed with TGF-β to fresh T cells on IgG production. The horizontal line indicates the starting cell number of $CD4^+$ cells (*A* and *B*) or IgG production (*C*). These experiments have been repeated five to eight times with similar results.

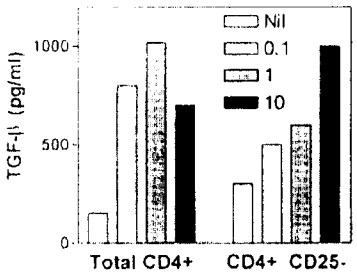

FIGURE 10. Comparison of active TGF-β production by total CD4+ and CD4+ CD25+ cells. Total CD4+ cells or CD4+CD25+ cells (2 × 10⁵/well) and SPC were stimulated with low-dose SEB and graded concentrations of TGF-β for 6 days; after extensive washing they were restimulated with SEB for 72 h. Values for active TGF-β are shown. This experiment was repeated four times with similar results.

SEB. A role for TGF-β in regulating the low-dose response to SEB (0.01–1 ng/ml) was initially suggested by an inverse correlation between IgG production and TGF-β produced by PBL and PBMC. Whereas IgG production by purified CD4+ and B cells remained high in this dose range, these lymphocytes were poor producers of TGF-β. Studies with a neutralizing anti-TGF-β Ab provided further evidence for regulation of IgG production by TGF-β.

The finding that anti-TGF-β needed to be present at the start of the culture for enhancement of Ab production (Fig. 2) suggested that this cytokine was regulating early T cell activation rather than B cell differentiation. Previously, we had documented that TGF-β needed to be present at the time of T cell activation for the generation of CD8+ regulatory T cells (4). While initial experiments revealed TGF-β down-regulated helper activity provided by CD4+ cells, further studies indicated that TGF-β induced CD4+ cells to inhibit IgG production by producing suppressive levels of this cytokine. An autocrine effect of TGF-β has been documented by others (17).

Previously, Stohl and Elliott (8) demonstrated that the T cell response to SEB is dose dependent. Whereas high-dose SEB resulted in T cell killing of B cells, low-dose SEB stimulated T cell-dependent B cell differentiation. Similar dose-dependent regulatory effects have been described in oral tolerance. High doses of Ag administered orally result in the clonal deletion of the responding T cells, whereas low doses of Ag result in the appearance of TGF-β-producing regulatory T cells called Th3 cells (18).

The Th3 cells described by Weiner (32) produced large amounts of TGF-β and some IL-10. Studies on the cytokines produced by our TGF-β-conditioned CD4+ cells revealed that TGF-β, but not IFN-γ, IL-10, or IL-4, was made in large amounts. Production of IL-10 and IFN-γ was less than that of SEB-stimulated control CD4+ cells. The decreased amount of IFN-γ was a direct effect of TGF-β, consistent with the findings of others (19). By contrast, the levels of IL-10 did not increase in the presence of anti-TGF-β.

To begin to understand the mechanism of action of TGF-β, we documented costimulatory effects of this cytokine on CD4+ cells. In preparations containing both CD4+ and CD8+ T cells, TGF-β selectively costimulated CD4+ cells in primary cultures, and this subset expanded preferentially upon restimulation without this cytokine. The expansion of T cells primed with TGF-β after restimulation has been described previously (14). In mice, one group has reported that TGF-β had selective costimulatory effects on CD8+ T cells (15). Proliferation in response to anti-CD3 and SEB was substantially enhanced by TGF-β (20). These treated CD8+ cells secreted IL-10 and TGF-β and developed cytokine-dependent growth inhibitory activity. We also have demonstrated costimulatory effects of TGF-β on purified human CD8+ T cells (3, 4) and have used TGF-β to generate regulatory CD8+ T cells that have cytokine-mediated suppressive effects (21). CD4+ cells and CD8+ cells require a different length of exposure to TGF-β for the induction of suppressive activity. Whereas incubation of CD8+ cells with TGF-β for 24 h is sufficient, 5–6 days of incubation were required for CD4+ cells to develop suppressive effects (3–5). Thus, TGF-β may have costimulatory properties upon either CD4+ or CD8+ cells, and a preferential effect on one subset may be explained by the experimental conditions used.

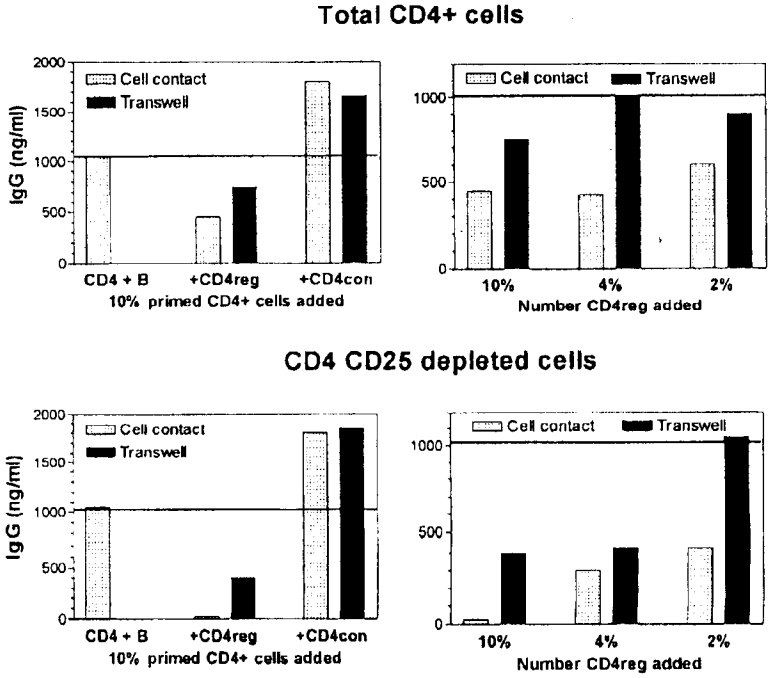

FIGURE 11. Role of cell contact in suppression of CD4+ cells conditioned with TGF-β. CD4reg or CD4con were prepared from total CD4+ cells (*upper panels*) or CD4+CD25+ cells (*lower panels*) (see *Materials and Methods*). Various percentages of these TGF-β-primed CD4+ cells were added to fresh CD4 and B cells (5 × 10⁴/well) or to Transwells that also contained SPC. The cells were stimulated with low-dose SEB for 10 days and IgG was measured. The result shown is representative of three experiments.

The next series of experiments revealed that priming $CD4^+$ cells with TGF-β not only enhanced the proliferation of $CD4^+$ cells upon restimulation but also protected these cells from activation-induced apoptosis. Others have reported that the combination of IL-2 and TGF-β has a similar protective effect on mouse $CD4^+$ T cells (22). This decreased apoptosis can be explained by decreased expression of Fas ligand (23, 24) and increased expression of mitochondrial Bcl-x_L (25).

Recently, we have reported other evidence of costimulatory effects of TGF-β on alloantigen-activated $CD4^+$ T cells. Similar to the results described in the present study, there was increased blast transformation, increased expression of CD25 and CTLA-4, and potent, contact-dependent suppressive effects on $CD8^+$ cells (5). The phenotype and functional properties of these $CD4^+$ cells were similar to, if not identical with, the professional $CD4^+CD25^+$ T cells described by others where neutralizing anti-cytokine Abs had no effect on suppressive activity (reviewed in Ref. 7). Here the $CD4^+$ cells produced large amounts of TGF-β, and neutralizing Abs to this cytokine abrogated suppressive activity.

Because we had previously generated $CD4^+$ suppressor cells with a cytokine-independent mechanism of action from $CD25^-$ precursors, we considered that the Th3 suppressor cells described in the present study were derived from $CD25^-$ resting $CD4^+$ cells. Subsequent studies revealed that the phenotype and function of cells generated from $CD4^+CD25^+$ cells were indistinguishable from those derived from total $CD4^+$ cells. Others have also documented suppressive properties of cells generated from the $CD4^+CD25^-$ fraction (26, 27). It is important to emphasize that the CD25 marker cannot distinguish "professional" regulatory cells from Th3 cells. Although the former constitutively express this marker, the latter also can display this determinant after T cell activation. Whereas "professional" $CD4^+CD25^+$ cells appear to be a unique lineage of thymic-derived T cells (6, 7), Th3 cells develop in the periphery (18).

While some workers claim that TGF-β has no role in the suppressive effector activity of $CD4^+CD25^-$ cells (7), others claim that this activity is abolished by high concentrations of anti-TGF-β and that latent TGF-β is bound to the surface of these cells (28). It is possible that heterogeneous populations of $CD4^+CD25^+$ cells derived from different precursors explain these apparently contradictory observations.

Both the thymus-derived $CD4^+CD25^+$ cells and Th3 cells generated from $CD25^-$ precursors in the periphery have important regulatory functions in vivo. The former block the activation of potentially aggressive, self-reactive T cells not eliminated by the thymus which are capable of causing systemic autoimmune disease (6, 7, 29, 30). $CD4^+CD25^+$ cells also regulate homeostatic T cell expansion (31). While Th3 cells can also suppress autoimmunity, they function principally as general feedback regulators of Th1 and Th2 cells (18, 32). Moreover, it is possible that these populations interact with each other in a synergistic manner. The ability to generate each of these populations ex vivo with TGF-β has the potential to be useful in the treatment of autoimmune diseases and prevention of transplant rejection.

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